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The use of kinin B_1 and B_2 receptor knockout mice and selective antagonists to characterize the nociceptive responses caused by kinins at the spinal level

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Abstract

The mechanisms by which kinins induce hyperalgesia in the spinal cord were investigated by using B_1 or B_2 knockout mice in conjunction with kinin selective agonists and antagonists. The i.t. administration of the kinin B_2 receptor agonists, bradykinin (BK) or Tyr^8-BK produced dose-related thermal hyperalgesia evaluated in the hot-plate test. BK-induced hyperalgesia was abolished by the B_2 receptor antagonist Hoe 140. The i.t. injection of the kinin B_1 receptor agonists, des-Arg^9-bradykinin (DABK) or des-Arg^{10}-kallidin (DAKD) also caused dose-related thermal hyperalgesia. Different from the B_2 agonists, the i.t. injection of DABK or DAKD caused a weak, but prolonged hyperalgesia, an effect that was blocked by the B_1 receptor antagonist des-Arg^9-[Leu^8]-bradykinin (DALBK). The i.t. injection of BK caused thermal hyperalgesia in wild-type mice (WT) and in the B_1 receptor knockout mice (B_1R KO), but not in the B_2 receptor knockout mice (B_2R KO). Similarly, the i.t. injection of DABK elicited thermal hyperalgesia in WT mice, but not in B_1R KO mice. However, DABK-induced hyperalgesia was more pronounced in the B_2R KO mice when compared with the WT mice. The i.t. injection of Hoe 140 or DALBK inhibited the second phase of formalin (F)-induced nociception. Furthermore, i.t. Hoe 140, but not DALBK, also inhibits the first phase of F response. Finally, the i.t. injection of DALBK, but not of Hoe 140, inhibits the long-term thermal hyperalgesia observed in the ipsilateral and in contralateral paws after intraplantar injection with complete Freund's adjuvant. These findings provide evidence that kinins acting at both B_1 and B_2 receptors at the spinal level exert a critical role in controlling the nociceptive processing mechanisms. Therefore, selective kinin antagonists against both receptors are of potential interest drugs to treat some pain states.

Keywords: Kinin; B_1 and B_2 receptor; Knockout mice; Hyperalgesia; Spinal cord

1. Introduction

Kinins are peptides formed in plasma and peripheral tissues in response to the activation of a class of enzymes denoted kallikreins acting on kininogen substrates. Kinins elicit a wide range of physiological effects including control of blood pressure, smooth muscle contraction or relaxation, vascular permeability and pain transmission mechanisms. Furthermore, kinins are implicated in some pathological states such as rheumatoid arthritis, pancreatitis, asthma and endotoxic shock (for review see: Marceau et al., 1998; Calixto et al., 2000, 2001). The actions of kinins are mediated through stimulation of two subtypes of seven transmembrane G-protein coupled receptors, denoted B_1 and B_2. The kinin B_1 receptors exhibit higher affinity for the kinin active carboxypeptidase metabolites des-Arg^9-bradykinin (DABK) and des-

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Arg[10]-kallidin (DAKD). The B_1 receptors are normally rarely expressed in non-traumatised tissues, but they can be expressed under certain particular conditions, such as following tissue injury and infection (Marceau et al., 1998; Calixto et al., 2000, 2001). On the other hand, bradykinin (BK) and kallidin (KD) exhibit great affinity for the B_2 receptors, which are normally constitutive and widely distributed throughout central and peripheral tissues. Thus, most of the physiological kinin actions seem to be mediated by stimulation of constitutive B_2 receptors (for review see: Marceau et al., 1998).

Once formed in the periphery, kinins activate Aδ and C fibres in sensory nerves producing pain, hyperalgesia or allodynia both in humans and experimental animals. In addition, kinins may have some indirect effects mediated by release of pro-inflammatory and hyperalgesic mediators such as neuropeptides neurokinins and calcitonin gene-related peptide (CGRP), nitric oxide and metabolites derived from the arachidonic acid pathway, which also largely accounts for their pro-inflammatory and nociceptive properties (see for review: Calixto et al., 2000, 2001; Campos and Calixto, 2000; Dray and Perkins, 1997).

In spite of their well-known peripheral localisation and effects for kinins, the actions of these peptides in the central nervous system (CNS), which contain all of the components of the kallikrein-kinin system, still remains controversial (Couture and Lindsey, 2000; Hall and Geppetti, 1995; Raidoo and Bhoola, 1998; Walker et al., 1995). A high density kinin binding site is in the CNS has been identified mainly in cerebral blood vessels. However, both B_1 and B_2 receptors have been identified in the dorsal root ganglion (DRG) and in the superficial layers of the dorsal horn, confined mainly to the terminals of primary sensory nerve fibres of the spinal cord (Steranka et al., 1988; Seabrook et al., 1997; Couture and Lindsey, 2000; Wotherspoon and Winter, 2000). Moreover, the intrathecal injection of bradykinin in mice causes pain-related behaviour (Hitosugi et al., 1999) and thermal hyperalgesia (Kamei et al., 2000) suggesting a role for B_2 receptors in the modulation of nociceptive process as in the spinal cord.

Recently, the development of molecular biology has permitted the generation of both B_1 and B_2 knockout mice, and this has largely accounted for the understanding of the role played by kinins in most physiological and pathological processes (Rupniak et al., 1997; Seabrook et al., 1997; Pesquero et al., 2000; Araújo et al., 2001). The deletion of the B_1 receptor gene significantly decreases the nociception caused by capsaicin, formalin, complete Freund's adjuvant and high thermal stimulus (Pesquero et al., 2000; Ferreira et al., 2001). Apart from the several studies showing an antinociceptive effect of B_2 receptor antagonists in various chemical models of nociception (Burgess et al., 2000; Corrêa and Calixto, 1993), mice lacking the B_2 receptor gene showed reduced thermal hyperalgesia only in response to carrageenan (Rupniak et al., 1997). Furthermore, it has been reported that gene deletion of B_2 receptor causes an increase in the density of B_1 receptors in the DRG and in the spinal cord (Seabrook et al., 1997; Couture and Lindsey, 2000).

Although kinins are the subject of much recent research and in spite of the available evidence indicating an important role for kinins in pain transmission and inflammatory states, thus far, there is no comparative study between the two classes of knockout mice following selective agonists stimulation. Therefore, in the present study we use the B_1 or B_2 knockout mice in conjunction with selective kinin receptors agonists and antagonists to evaluate the contribution of spinal cord B_1 and B_2 receptors for kinins in acute, sub-chronic and chronic models of nociception.

2. Material and methods

2.1. Animals

Experiments were conducted using male Swiss, or male and female 129/J, wild-type, B_1 or B_2 kinin receptor knockout mice (20–30 g) kept in controlled room temperature (22 ± 2 °C) under a 12 h:12 h light-dark cycle (lights on 06:00 h). Swiss mice were obtained at the Department of Pharmacology, Universidade Federal de Santa Catarina (UFSC, Florianópolis, Brazil). Wild type J129, B_1 or B_2 kinin receptor knockout mice were obtained at the Department of Biophysics, Escola Paulista de Medicina (EPM-UNIFESP, São Paulo, Brazil). Deletion of the entire coding sequence for B_1 and B_2 kinin receptors was achieved according to the methodology described previously by Pesquero et al. (2000) and Rupniak et al. (1997), respectively. The experiments were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals (Zimmermann, 1983). The number of animals and intensity of noxious stimuli were the minimum necessary to demonstrate consistent effects of drug treatments or genetic manipulation (Rupniak et al., 1997).

2.2. Intrathecal (i.t.) injections

The i.t injections were performed in accordance with the method described by Hylden and Wilcox (1980), with minor modifications. The animals were anaesthetised lightly with ether, and a volume of 5 μl of phosphate buffer solution (PBS, composition mmol/L: NaCl 137, KCl 2.7 and phosphate buffer 10) alone (control) or containing the drugs, was injected between L5 and L6 using a microsyringe connected to polyethylene tubing as described previously (Ferreira et al., 1999).

2.3. Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described by Woolfe and MacDonald (1944), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37) was maintained at 50 ± 1 °C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placement and shaking or licking of the paws, or jumping, was recorded as the index of response latency. An automatic 30 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline.

2.4. Kinins-induced thermal hyperalgesia

Experiments were carried out as described previously (Ferreira et al., 1999). Following PBS, bradykinin, Tyr^8-bradykinin, des-Arg^9-bradykinin or des-Arg^{10}-kallidin (1-50 nmol/site) administration into mice, the hot-plate latencies were recorded at several time-points (10, 20, 30, 60, 90 and 120 min) in absence and in presence of selective kinin B_1 (des-Arg^9-[Leu^8]-bradykinin, DALBK) or B_2 (icatibant, Hoe 140) receptor antagonists. The peak of hyperalgesic response caused by i.t. injection of B_1 and B_2 kinin receptor agonists was observed at 60 or 10 min after the i.t. administration of the peptide, respectively.

To confirm the involvement of B_1 and B_2 receptors in the kinins-induced thermal hyperalgesia, BK (5 nmol/site) or DABK (50 nmol/site) were administered in wild-type or in B_2 or B_1 receptor knockout mice. The maximal possible effect (MPE) of kinins-induced hyperalgesia was calculated as follows: %MPE = [(postdrug − predrug)/(30 − predrug)] × 100. The %MPE was estimated for each mouse, using 6–8 animals per dose. When possible, the dose response curves were performed using at least three doses of tested drugs.

To test the intrathecal hyperalgesic effect of kinins (BK, DABK, Tyr^8-BK, DAKD), animals were pretreated with the angiotensin-converting enzyme inhibitor, captopril (5 mg/kg, s.c.) 1 h before the intrathecal injection in order to prevent degradation of the peptides administered (Campos and Calixto, 1995).

2.5. Formalin-induced licking

The procedure was similar to that described previously (Corrêa and Calixto, 1993). Twenty μl of 2.5% formalin (0.92% formaldehyde) made up in PBS, was injected under the plantar surface of the right hind paw of Swiss mice. Animals were treated with Hoe 140 (3–30 pmol/site) or with DALBK (3–30 pmol/site) by i.t. route, 10 min before the formalin injection. Control animals received only the vehicle used to dilute the antagonists (PBS, 5 μl/site). Following an intraplantar injection of formalin, animals were immediately placed into a glass cylinder of 20 cm in diameter, and the time spent licking the injected paw, was timed with a chronometer and considered as indicative of nociception.

2.6. CFA-induced thermal hyperalgesia

The paw withdrawal latencies were used to measure thermal hyperalgesia according to the method described by Hargreaves et al. (1988). Swiss mice were placed in clear plastic chambers (7 × 9 × 11 cm) on an elevated surface and allowed to acclimatise to their environment for 1.5 h before testing. The heat stimulus was directed to the plantar surface of each hind paw in the area immediately proximal to the toes. The infra-red intensity was adjusted to obtain basal paw withdrawal latencies of about 11 sec. An automatic 20 sec cut-off was used to prevent tissue damage. Mice were slightly anaesthetised with ether and received a 20 μl intradermal (i.d.) injection of CFA (1 mg/ml of heat killed *Mycobacterium tuberculosis* in paraffin oil 85% and mannide monoleate 15%) into right hind paw. In order to analyse the possible contralateral effect of CFA injection, the left paw did not receive any injection. After challenge, hyperalgesia was measured 6 h after CFA i.d. injection, as described below (Ferreira et al., 2001). Animals were treated with PBS (5 μl/site, control group), Hoe 140 (10 pmol/site) or with DALBK (100 pmol/site) by i.t. route, 10 min before the hyperalgesia measure.

2.7. Drugs

The following drugs were used: Complete Freund's adjuvant (CFA; H37Ra, ATCC, 25177), bradykinin, Tyr^8-bradykinin, des-Arg^9-bradykinin, des-Arg^{10}-kallidin, des-Arg^9-[Leu^8]-bradykinin, captopril, PBS tablets, all from Sigma Chemical Company (St. Louis, USA); and formalin from Merck A.G. (Darmstadt, Germany). Hoe 140 (icatibant) was kindly provided by Aventis (Frankfurt am Main, Germany). The stock solutions of the peptides were prepared in PBS in siliconised plastic tubes, maintained at −18 °C and diluted to the desired concentration just before use.

2.8. Statistical analysis

The results are presented as the mean ± SEM of 4–6 animals. Statistical significance of differences between groups was assessed by means of one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multicomparison test. P values < 0.05 were considered significant. When possible, the ED_{50} or ID_{50} values (i.e. the dose of kinins producing half maximal hyperalgesic response or the dose of antagonists that reduced the hyp-

eralgesic or nociceptive responses by 50% of response relative to control value) were determined in each individual experiment at the peak of hyperalgesic or nociceptive response. The ED_{50} or ID_{50} values were determined by graphical interpolation from individual experiments by use of at least three doses of each agonist or antagonist, and are reported as geometric means accompanied by their respective 95% confidence limits. We also calculated the MPE or MPI values (i.e. the maximal percentage of hyperalgesic effect or inhibition, respectively). In order to obtain data purely derived by the treatments in kinin-induced hyperalgesia, the maximal inhibition (MI) values were represented as the difference between the %MPE of PBS- and kinin-treated animals.

3. Results

3.1. Effects of exogenously-administered kinins on the thermal stimulus sensitivity

The i.t. administration of BK or Tyr^8-BK to mice (1–30 nmol/site) resulted in a dose-related thermal hyperalgesia in captopril pre-treated mice, evaluated in the hot-plate test maintained at 50 °C (Fig. 1A–D). In the dose used, pre-treatment of mice with captopril (5 mg, kg, s.c.) did not produce any detectable effect per se on the baseline latencies (results not shown). This hyperalgesic effect installed quickly, peaked at 10 min and lasted for up to 60 min. The calculated mean ED_{50} values (and the 95% confidence limits) for BK- and Tyr^8-BK-induced hyperalgesia were 3.8 (3.1–4.7) and 6.5 (5.4–7.8) nmol/site, respectively. The maximal percentages of BK- and Tyr^8-BK-induced hyperalgesia obtained for the higher doses used (30 nmol per site) were 67 ± 10% and 42 ± 3%, respectively.

The i.t. administration of the selective B_2 receptor antagonist, Hoe 140 (1–10 pmol/site), did not induce any detectable effect per se on the baseline, but instead produced dose-related inhibition of BK-induced hyperalgesia (Fig. 2A). The calculated mean ID_{50} value for this effect (and their respective 95% confidence limits) was: 4.8 (4.1–5.6) pmol/site. Moreover, the selective B_1 receptor antagonist DALBK (25 nmol/site) did not produce any detectable effect at the baseline, nor did it inhibit the early stage (10–20 min) of BK-induced hyperalgesia. However, DALBK partially, but significantly, inhibited the late stage (30–60 min) of BK-induced hyperalgesia (Fig. 2B).

BK-induced hyperalgesia was absent in the B_2 kinin receptor knockout mice, when compared with the wild-

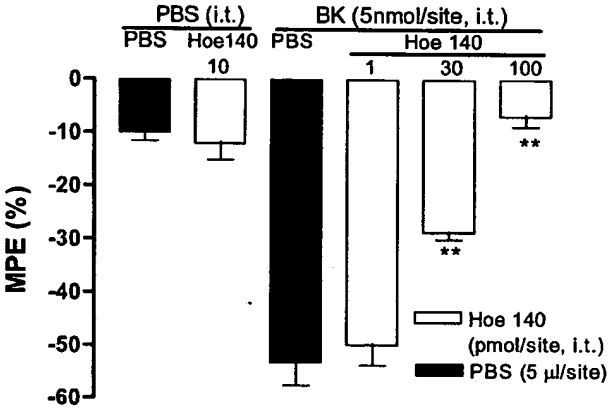

Fig. 2. Effect of the co-administration of the selective B_2 kinin receptor antagonist Hoe 140 on the hyperalgesia assessed 10 min after the intrathecal injection of bradykinin (BK) in mice. The effects of the drugs are expressed as MPE (%). Each column represents the mean of six animals and the vertical lines show the SEM. Asterisks denote the significance levels in comparison with control (BK or DABK-treated group) values (one-way ANOVA followed by Dunnett's test). $*P < 0.05$, $**P < 0.01$.

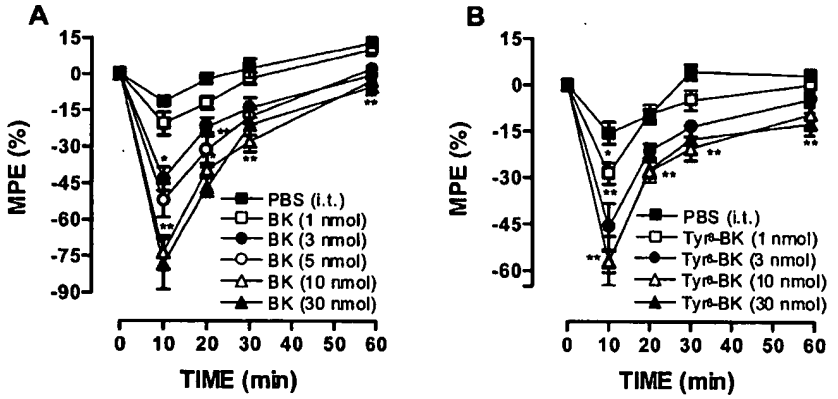

Fig. 1. Time-course and dose-response curves for the hyperalgesic action caused by intrathecal injection of the B_2 kinin receptor agonists bradykinin (BK, A) and Tyrosine^8-bradykinin (Tyr^8-BK, B) in mice. The effects of the drugs are expressed as MPE (%). For dose-response curves the MPE (%) was calculated at the hyperalgesia peak (10 min). Each point on the curve or column represents the mean of six to eight animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison with control (PBS-treated group) values (one-way ANOVA followed by Dunnett's test). $*P < 0.05$, $**P < 0.01$. In some cases the error bars are hidden within the symbols.

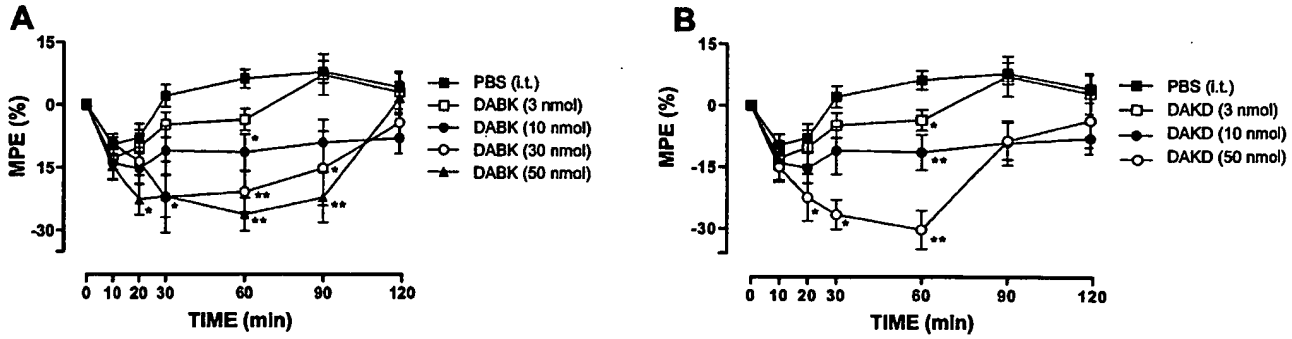

Fig. 3. Time-course and dose-response curves for the hyperalgesic action caused by intrathecal injection of the B_1 kinin receptor agonists des-Arg^9-bradykinin (DABK, A) and des-Arg^{10}-kallidin (DAKD, B) in mice. The effects of the drugs are expressed as MPE (%). For dose-response curves the MPE (%) was calculated at the hyperalgesia peak (60 min). Each point on the curve or column represents the mean of six to eight animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison with control (PBS-treated group) values (one-way ANOVA followed by Dunnett's test). $*P < 0.05$, $**P < 0.01$. In some cases the error bars are hidden within the symbols.

type mice (Fig. 3A). Moreover, no differences were detected in the early stage (10–20 min) of BK-induced hyperalgesia, in the B_1 receptor deficient mice or in the wild-type mice. The deletion of B_1 receptor gene reduced partially, but significantly, the late stage (30–60 min) of BK-induced hyperalgesia (Fig. 3B).

The i.t. administration of the selective B_1 agonists DABK or DAKD to mice (3–50 nmol/site) resulted in a dose-related thermal hyperalgesia, assessed in the hot-plate test (Fig. 4A and B). This hyperalgesic effect installed slowly, was significant at 20 min, peaked at 60 min and lasted for up to 90 min. The calculated mean ED_{50} values (and the 95% confidence limits) for DABK or DAKD-induced hyperalgesia were 21.3 (14.8–30.7) and 22.7 (17.0-30.4) nmol/site, respectively. The maximal percentage of DABK or DAKD-induced hyperalgesia obtained for the higher dose used (50 nmol per site) were 32 ± 4% and 37 ± 5%, respectively.

The i.t. administration of the selective B_1 receptor antagonist, DALBK (1–25 nmol/site), did not induce any detectable effect per se on the baseline, but instead caused dose-related inhibition of DABK-induced hyperalgesia (Fig. 5A). The calculated mean ID_{50} value (and their respective 95% confidence limits) was 5.8 (2.8–12.0) nmol/site.

DABK-induced hyperalgesia was absent in the B_1 kinin receptor knockout mice, when compared with the wild-type mice (Fig. 6A). Unexpectedly, DABK-induced hyperalgesia was significantly more pronounced in the B_2 receptor deficient mice, when compared with the wild-type mice (Fig. 6B).

3.2. *Effects of intrathecally-administered kinins receptor selective antagonists on the formalin-induced licking*

The i.t. pre-treatment (10 min prior formalin injection) with the selective B_2 receptor antagonist Hoe 140 (10–300 pmol/site) produced a dose-related inhibition of the early and late phase of the formalin-induced licking (Fig. 7A and B). The inhibitions observed were 23 ± 5% and 51 ± 4%, for the early and late phase, respectively. The intrathecal pre-treatment (10 min prior formalin injection) with the selective B_1 receptor antagonist

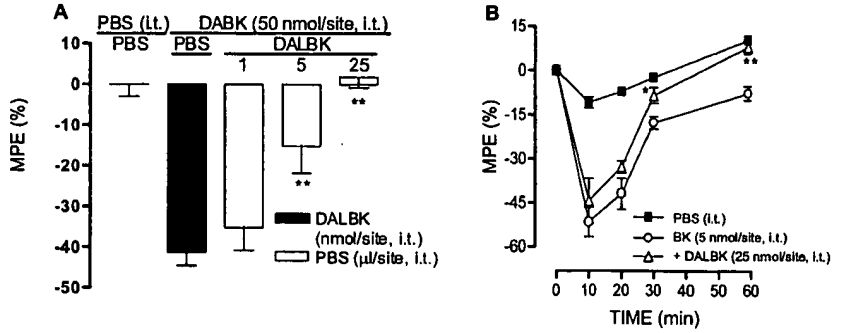

Fig. 4. Effect of the co-administration of the selective B_1 kinin receptor antagonist des-Arg^9-Leu^8-bradykinin (DALBK) on the hyperalgesia assessed 60 min after the intrathecal injection of des-Arg^9-bradykinin (DABK, A) or assessed 10 min after the intrathecal injection of bradykinin (BK, B) in mice. The effects of the drugs are expressed as MPE (%). Each point on the curve or column represents the mean of six animals and the vertical lines show the SEM. Asterisks denote the significance levels in comparison with control (BK or DABK-treated group) values (one-way ANOVA followed by Dunnett's test). $*P < 0.05$, $**P < 0.01$.

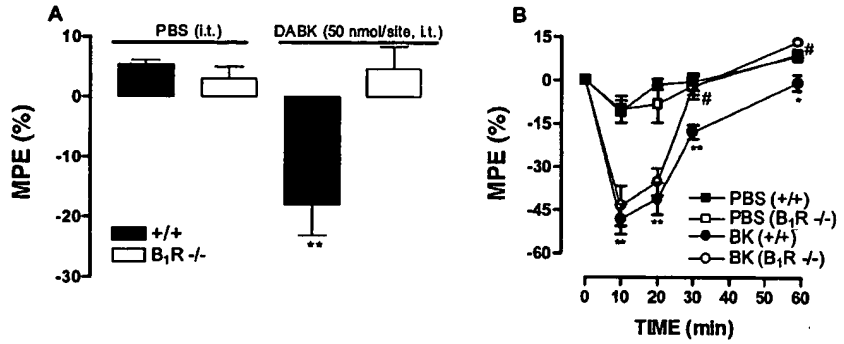

Fig. 5. Hyperalgesia caused by intrathecal injection of des-Arg^9-bradykinin (DABK, A) or bradykinin (BK, B) in wild type (+ / +) or B_1 kinin receptor knockout (B_1R −/−) mice. The hyperalgesic effects are expressed as MPE (%). Each point on the curve or column represents the mean of four animals and the vertical lines show the SEM. $*P$ or $\#P$ compared with PBS-treated wild type group or with BK- and DABK-treated wild type group, respectively (one-way ANOVA followed by Tukey's test). $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$.

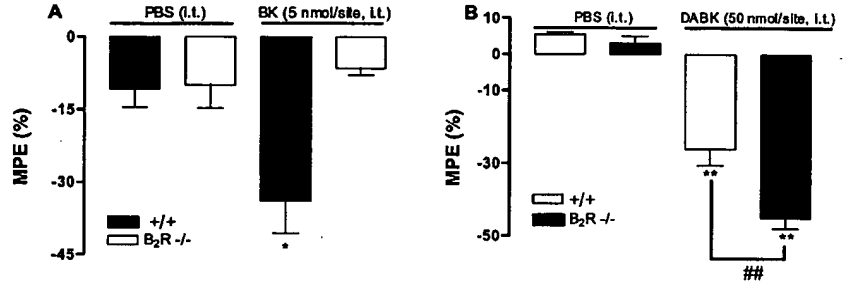

Fig. 6. Hyperalgesia caused by intrathecal injection of bradykinin (BK, A) or des-Arg^9-bradykinin (DABK, B) in wild type (+ / +) or B_2 kinin receptor knockout (B_1R −/−) mice. The hyperalgesic effects are expressed as MPE (%). Each column represents the mean of four animals and the vertical lines show the SEM. $*P$ or $\#P$ compared with PBS-treated wild type group or with BK- and DABK-treated wild type group, respectively (one-way ANOVA followed by Tukey's test). $*P < 0.05$, $**P < 0.01$, $\#\#P < 0.01$.

DALBK (3–30 nmol/site) inhibit the late, but not the early, phase of formalin-induced licking (Fig. 7C and D). The inhibition observed was 44 ± 8%.

Similar to the observations in the pre-treatment regimen, the i.t. post-treatment (5 min after formalin injection) with Hoe 140 (100 pmol/site) or with DALBK (10 pmol/site), also significantly inhibited the late phase of formalin-induced licking (mean licking time of 159 ± 11 s, 82 ± 14 and 55 ± 6 s for PBS, Hoe 140 and DALBK-treated mice, respectively). The inhibitions were 49 ± 9 and 65 ± 4%, for Hoe 140 and DALBK, respectively.

3.3. Effects of intrathecally-administered kinins receptor selective antagonists on the CFA-induced thermal hyperalgesia

As reported previously (Ferreira et al., 2001) the i.d. injection of CFA into mice paws produced a time-dependent and marked thermal hyperalgesia. These responses were represented by a significant reduction of paw withdrawal latencies to thermal stimuli observed in both ipsilateral and, to a lesser extent, in contralateral hindpaws (Fig. 8A and B). The i.t. post-treatment (6 h after CFA injection) with the selective B_1 receptor antagonist DALBK (10 pmol/site) inhibited both, ipsilateral and contralateral thermal hyperalgesia (Fig. 8A and B). The inhibitions observed were 62 ± 17% and 100%, for ipsilateral and contralateral hyperalgesia, respectively. However, the intrathecal post-treatment with the selective B_2 receptor antagonist Hoe 140 (100 pmol/site) failed to inhibit either the ipsilateral or the contralateral hyperalgesia induced by CFA (Fig. 8A and B).

4. Discussion

Insults in peripheral tissues, such as skin, joints and viscera, release several algogenic mediators that in turn activate nociceptors that sensitise for other painful stimulus. Activation of nociceptors in the periphery produces messages that converge in the dorsal horn of the spinal cord, where nociceptive information is processed, and projected to the cerebral cortex via a relay in the thalamus, eliciting pain (Woolf and Salter, 2000). There is convincing evidence from pharmacological, electrophysiological and molecular biology studies, that kinins, acting in peripheral B_1 or B_2 receptors, play a critical role in controlling the painful processes (see for recent review Calixto et al., 2000, 2001; Dray and Perkins,

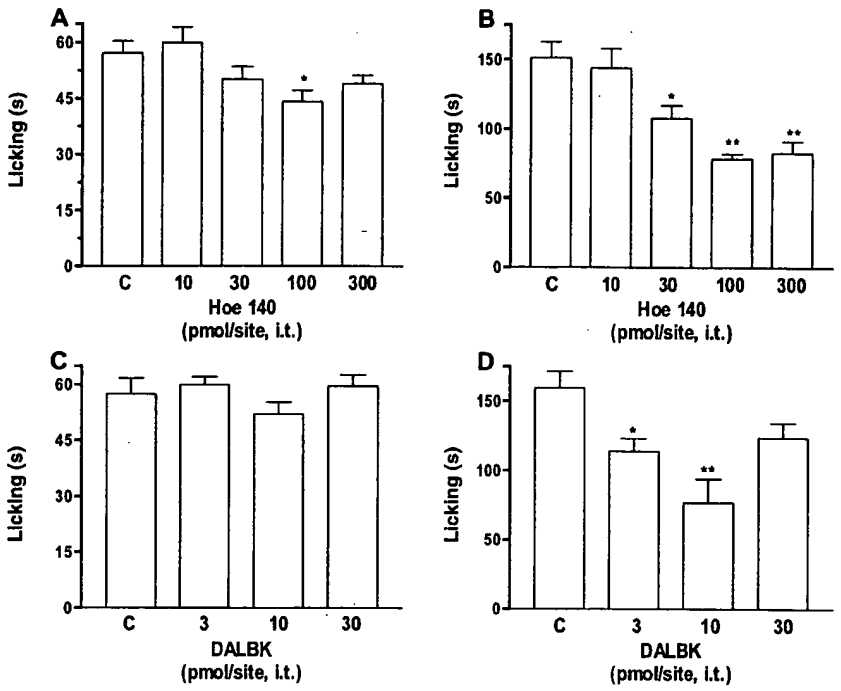

Fig. 7. Effect of intrathecal pre-treatment (10 min before irritant injection) with the selective B_2 (Hoe 140, A and B) and B_1 (DALBK, C and D) receptor antagonists on first (A and C) or second phase (B and D) of formalin-induced nociception in mice. Each column represents the mean of six animals and the vertical lines show the SEM. C indicates control (PBS-treated) animals. Asterisks denote the significance levels in comparison with control values (one-way ANOVA followed by Dunnett's test). $*P < 0.05$, $**P<0.01$.

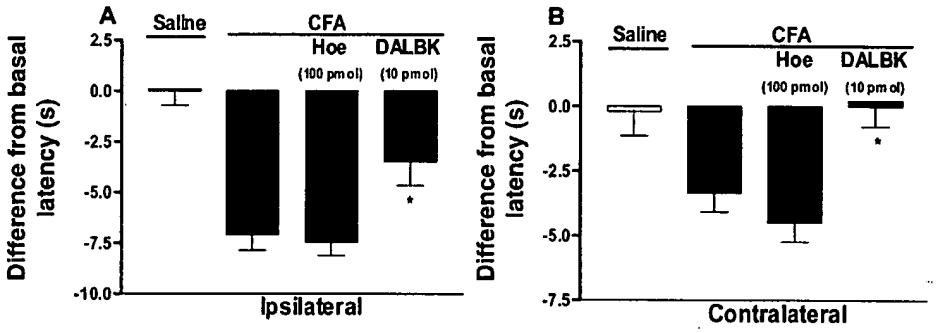

Fig. 8. Effect of intrathecal post-treatment (6 h after irritant injection) with the selective B_2 (Hoe 140) and B_1 (DALBK) receptor antagonists on CFA-induced thermal hyperalgesia in ipsilateral (A) and contralateral (B) paws of mice. Each column represents the mean SEM of four mice. The asterisks denote the significance levels. $*P < 0.05$ compared with PBS-treated mice (one-way ANOVA followed by Dunnett's test).

1997). As well as these peripheral effects, several in vitro and in vivo studies suggest that kinins may participate in pain transmission at the spinal level. In vitro studies have identified B_1 and B_2 receptors in the spinal cord terminals of sensory neurones (Couture and Lindsay, 2000; Steranka et al., 1988; Wotherspoon and Winter, 2000). Thus, B_1 and B_2 selective agonists depolarise neurones in spinal cord preparations (Dunn and Rang, 1990; Pesquero et al., 2000) and induce neuropeptide release (Andreeva and Rang, 1993; Wang and Fiscus, 1997). In addition, intrathecal injection of BK causes pain-related behaviour (Hitosugi et al., 1999) and thermal hyperalgesia (Kamei et al., 2000) in mice, and intrathecal administration of the B_2 receptor antagonist Hoe 140 reduces the second phase of formalin nociception in rats (Chapman and Dickenson, 1992). Here we used B_1 and B_2 knockout mice in conjunction with selective agonists and antagonists for both receptors to further characterise the mechanisms by which kinins induce hyperalgesia when injected intrathecally to mice.

Results reported in the present study confirm and extend previous data by demonstrating the critical role exerted by kinins in transmission of nociceptive information in the spinal cord of mice. Thus, exogenous administration of B_1 and B_2 selective agonists into the spinal cord of mice produces significant dose-related

thermal hyperalgesia. However, it was noted that there were marked differences between their hyperalgesic responses. While i.t. injection of BK or the selective B_2 agonist Tyr^8-BK produced a strong and long-lasting hyperalgesia which installed quickly, peaked at 10 min and last for up to 60 min, the selective B_1 agonists DABK and DAKD elicited a less intense, but low onset hyperalgesic response that peaked at 60 min and lasted for up to 90 min. At the ID_{50} level, the selective B_2 agonists were about 3–6 fold more potent compared with B_1 selective agonists. A great amount of functional pharmacological and molecular studies have confirmed that both B_1 and B_2 receptors are two separate entities. The B_2 receptors exhibit high affinity for bradykinin and kallidin and are rapidly desensitised and internalised. In contrast, the B_1 receptors bind preferentially to the kinin metabolites des-Arg^9-BK and des-Arg^{10}-kallidin and are not desensitised or internalised (Marceau et al., 1998; Calixto et al., 2001). When compared with the B_2 receptor activation, the B_1 receptor activation causes much more prolonged intracellular signals, such as increases in the intracellular calcium concentration. These characteristics may explain, at least in part, the differences between B_1 and B_2 receptor agonist responses observed in the present study.

The confirmation of spinal kinin receptor subtypes involved in these effects derives from the findings showing kinin-induced hyperalgesia in animals with deletion of the gene for kinin B_1 or B_2 receptors in conjunction with i.t. administration of selective kinin receptor antagonists. Thus, in mice lacking the B_2 receptor gene, in contrast with wild-type littermates, the i.t. injection of BK did not exhibit any hyperalgesic response. Likewise, the i.t. injection of the selective B_2 receptor antagonist Hoe 140, inhibited dose-dependently, and completely BK-induced hyperalgesia. Although it has long been recognised that the B_2 kinin receptor plays an important role in the process of pain transmission, little attention has been given to the participation of the B_1 receptor in this effect (see for review Calixto et al., 2000, 2001; Couture et al., 2001). The results of the present study show that the i.t. injection of the selective B_1 agonist DABK in mice lacking the B_1 receptor gene, did not elicit any hyperalgesic response when compared with similar experiments performed in wild type mice. Likewise, the i.t. injection of the selective B_1 receptor antagonist DALBK inhibited dose-dependently and completely, DABK-induced hyperalgesia. Moreover, the i.t. injection of DALBK reversed the late phase (30 and 60 min) of BK-induced hyperalgesia. Such results further confirm the notion that B_1 receptors account, at least in part, for the late phase of the hyperalgesia caused by i.t. injection of BK. Taken together, the present and the previous findings (Pesquero et al., 2000; Ferreira et al., 2001) provide consistent evidence indicating that B_1 receptors seem to exert a relevant role in kinin-induced hyperalgesia. It has been suggested that B_1 receptors are present in peripheral and spinal terminals and in DRG of sensory afferent fibres, and therefore participate in the process of pain transmission (Couture and Lindsey, 2000; Ma et al., 2000; Ma, 2001; Ma and Heavens, 2001; Seabrook et al., 1997; Steranka et al., 1988; Wotherspoon and Winter, 2000), although conclusive evidence for such a role is lacking (Dray and Perkins, 1997). However, Pesquero et al. (2000) using an in vitro functional preparation have shown that B_1 receptor activation increases the spinal cord reflex excitability in mice that would directly underlie a proalgesic role of this receptor in vivo. The results of the current study strongly indicate that the spinal cord represents a relevant site of action for kinins acting at B_1 and B_2 receptors.

Interestingly, the hyperalgesic response elicited by the selective B_1 agonist DABK was greatly potentated in B_2 knockout mice when compared with similar responses observed in wild type littermates. These results are in line with previous published studies (Couture and Lindsey, 2000; Ferreira et al., 2001; Seabrook et al., 1997), that showed an increase in B_1 receptor expression in the DRG neurones and spinal cord and also in the model of complete Freund's adjuvant-induced paw oedema in B_2 knockout mice. These data further support the notion that B_1 receptors are overexposed following deletion of the B_2 receptor gene (Maddedu et al., 1997; Duka et al., 2001). In fact, an auto-regulation of kinin receptor expression has been proposed in both in vivo (Campos and Calixto, 1995) and in vitro models (Phagoo et al., 1999). These authors have suggested the possible occurrence of a balance between the two populations of kinin receptors: while B_2 receptors mediate the acute phase of inflammatory responses, B_1 receptors are induced during chronic inflammatory states. Therefore, the complete absence of B_2 receptor function might determine the up-regulation of B_1 receptors.

Moreover, results obtained using i.t. injection of kinin receptor antagonists further support the concept that endogenous B_1 or B_2 receptor agonists are produced in spinal cord and their inhibition produces analgesia in models of acute, subchronic and chronic nociception. These findings strongly support the proposal that kinins acting at receptors in the spinal cord seem to control the process of pain transmission in vivo.

Confirming previous studies from this and other groups (Corrêa and Calixto, 1993; Hunskaar and Hole, 1987; Shibata et al., 1989), the intraplantar injection of formalin induces a pain-related behaviour with two distinct phases. The first phase is a measure of the direct activation of nociceptors and the second phase reflects ongoing peripheral input acting on dorsal horn neurones sensitised by the input arising in the first phase (Ji and Woolf, 2001). So, spinal kinin B_2 receptors are involved in both normal and early sensitised spinal nociceptive transmission. Our results support the concept that the B_2

kinin receptors seem to be activated in the spinal cord in the acute and sub-chronic nociceptive conditions (first and second phase of formalin test, respectively). On the other hand, spinal B_1 receptors are involved in early and late spinal sensitisation and seem to participate only in sub-chronic and chronic nociception transmission.

The results obtained with i.t. injection of kinin receptor antagonists in the CFA-induced chronic inflammation further confirm the notion that these receptors play an important role in central sensitisation during the development of hyperalgesia. In support of these findings, Pesquero et al. (2000) found a selective reduction in the use-dependent facilitation of the spinal cord neurones (windup) in B_1 receptor knockout mice, indicating that the sensory impairment observed in knockout B_1 receptor mice was attributed, at least in part, to a deficit in the pathological plasticity of the spinal neurones. On the other hand, spinal B_2 receptors do not seem to be involved in the windup phenomenon (Chapman and Dickenson, 1992). Moreover, the deletion of the B_1 receptor gene, but not of the B_2 receptor gene, reduces the thermal hyperalgesia caused by intraplantar injection of complete Freund adjuvant (Ferreira et al., 2001). Windup has been interpreted as a system for the amplification, in the spinal cord, of the nociceptive message that arrives from peripheral nociceptors connected to C-fibres. This probably reflects the physiological system activated in the spinal cord after an intense or persistent barrage of afferent nociceptive impulses (such as during inflammation) (Herrero et al., 2000).

In summary, we have demonstrated here, by using of B_1 and B_2 knockout mice in conjunction with selective B_1 and B_2 kinin agonists and antagonists, that kinins acting in both B_1 and B_2 receptors exert a critical role in the process of thermal spinal nociception. Together, these findings support the notion that the development of an oral selective B_1 receptor antagonist might be expected to have an improved therapeutic potential in the management of pain disorders.

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